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Tian, Hailin; Fotidis, Ioannis; Mancini, Enrico; Treu, Laura; Mahdy, Ahmed; Ballesteros, M.; González-Fernández, C.; Angelidaki, Irini

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H. Tian*, I. A. Fotidis*, E. Mancini*, L. Treu*, A. Mahdy**, M. Ballesteros***, C. González-Fernández*** and I. Angelidaki*

* Department of Environmental Engineering, Technical University of Denmark, Bygningstorvet Bygning 115, DK-2800 Kgs. Lyngby, DK
** Department of Agricultural Microbiology, Faculty of Agriculture, Zagazig University, 44511 Zagazig, EG
*** Biotechnological Processes for Energy Production Unit – IMDEA Energy, 28935 Móstoles, Madrid, ES
(E-mail: *ioanf@env.dtu.dk)*

Abstract
Anaerobic digestion (AD) is an effective technology to recover energy (CH_4) from biomass. However, when protein-rich by-products are used as feedstocks, they could lead to low methane yields and even to complete process failure. Acclimatized anaerobic communities to high ammonia levels can offer a solution to this problem. Nevertheless, the acclimation to high ammonia levels during continuous AD of protein-rich substrates still poses serious challenges (e.g. microbes can be washed out from reactors). In the present study, acclimation strategy followed by a stepwise increase (0.5 g NH_4^+-N L^{-1} each step) of total ammonia (TAN) concentration from 3.8 up to 10.5 g NH_4^+-N L^{-1} was investigated using continuous stirred tank reactors (CSTR) under mesophilic conditions (37±1°C). Microalgae *Chlorella vulgaris* (>50% protein in dry matter), mixed with cattle manure, was used as substrate. Throughout the acclimation period, even though the TAN increased 1.8-fold at the end of the experiment, the methane yield of the CSTR reactor was stable with an average variation less than 10% compared to the yield before ammonia acclimation started. At the same time, VFA and pH were both within the optimal range of AD process throughout the whole experimental period. These results demonstrated that an acclimation of ammonia tolerant methanogenic consortia to extreme ammonia levels using CSTR reactors is possible. Furthermore, microbiological analyses (16s rRNA sequencing) results confirmed a profound decrease in the microbial diversity due to the acclimation process.

Keywords
Ammonia inhibition; anaerobic digestion; CSTR reactor; methane; microalgae.

INTRODUCTION

Anaerobic digestion (AD) is a renewable technology that can be used for the treatment of organic biomass with simultaneous production of both energy production (CH_4) and fertilizer. Many full scale biogas plants are manure-based and in order to increase the methane yield of manure, and thereby improve profitability, they are co-digesting with proteinaceous substrates, such as slaughterhouse or food waste (Mata-Alvarez et al., 2000). Low ammonium levels (200 mg NH_4^+-N L^{-1}) are needed for an optimal microbial growth (Liu & Sung, 2002), while high ammonium loads (> 3 g NH_4^+-N L^{-1}) can severely inhibit AD process and lower the potential methane yield by 30% (Nielsen & Angelidaki, 2008). Furthermore, free ammonia (FAN), which increases alongside with pH and temperature, is known to be the most toxic form to anaerobes (Massé et al., 2014). Therefore, many solutions (Yenigün & Demirel, 2013) have been proposed to solve the ammonia toxicity problem in AD reactors such as: dilution with water, air stripping, absorbing addition, low operational temperature, submersible microbial desalination cell and bioaugmentation. These methods could alleviate ammonia inhibition to a certain extent, but they are either economically unfeasible or far from practical applicability. However, there are evidences that acclimation of microbial consortia to high ammonia levels could provide a practical and cost-efficient method for protein-rich substrates' digestion (Yenigün & Demirel, 2013). Additionally, recent research also suggests that acclimatization of anaerobic consortia to high ammonia levels is possible in batch and fed-batch reactors (Tian et al., 2017). However, limited information can be found in the literature

about successful acclimation of methanogens to high ammonia levels during continuous AD of protein-rich substrates. Today there is a growing interest in using protein-rich microalgae as AD substrate, which can be classified as 3rd generation substrate since they do not compete with food supplies. Therefore, the aim of the present study was to assess the possibility of methanogenic populations acclimation to extremely high ammonia levels in a continuous reactor, and identify the microbial community dynamics using 16S rRNA gene sequencing.

MATERIAL AND METHODS

Inoculum and feedstock

The inoculum used in the present study was obtained from a mesophilic (37±1°C) biogas plant (Hashøj, Denmark). A mixture (VS/VS) of 20% cattle manure and 80% microalga (*Chlorella vulgaris*) were used as feedstock. *Chlorella vulgaris* was used as a protein-rich biomass. Cattle manure was collected from the same biogas plant, while *Chlorella vulgaris* (>50% protein in dry matter) was grown in mineral salt (MBBM-2N) medium in a raceway with continuous illumination at 25ºC. After sieving to throw away large particles, cattle manure was stored at -18°C and defrosted at 4°C before use. After cultivation and harvesting, microalgae biomass was pretreated according to the methodology previously described (Mahdy et al., 2015). The basic characterization of inoculum and substrates are presented in Table 1. Ammonium chloride (NH_4Cl) was used as ammonia source.

Table 1. Characteristics of the inoculum, cattle manure and microalgae biomass.

Parameter	Inoculum	Cattle manure	Microalgae
	value ± SD	value ± SD	value ± SD
Total solids-TS (g L^{-1})	33.20 ± 0.19	32.90 ± 0.02	26.54 ± 0.04
Volatile solids-VS (g L^{-1})	19.80 ± 0.18	23.00 ± 0.04	23.00 ± 0.03
Total Ammonium nitrogen-TAN (g NH_4^+-N L^{-1})	4.58 ± 0.02	1.10 ± 0.12	0.57 ± 0.06
Total Kjeldahl nitrogen-TKN (g N L^{-1})	5.01 ± 0.13	1.49 ± 0.01	2.37 ± 0.03
Volatile fatty acids-VFA (mg L^{-1})	76.08 ± 5.75	8936.97 ± 50.51	442.71 ± 11.43

Experimental setup

A lab-scale mesophilic (37±1°C) CSTR reactor with a hydraulic retention time (HRT) of 23 days and organic loading rate (OLR) of 1.95±0.10 g VS $L^{-1}d^{-1}$ was used. The system consisted of a glass vessel with 2.3 and 1.8 L total and working volume, respectively, an influent and an effluent bottle, a feeding peristaltic pump, an electrical heating jacket, a water displacement gas meter and two magnetic stirrers for the homogenization of substrate and mixing of the reactor. Start-up of the reactor with cattle manure, then mixed feedstock of cattle manure and microalgae was used. After steady state was observed, acclimation test was started with a stepwise increasing TAN (0.5 g NH_4^+-N L^{-1} each step) up to 10.5 g NH_4^+-N L^{-1} both inside the reactor and the feedstock.

Analyses

Physicochemical analysis. TS, VS, TAN and TKN were measured through standard method. The pH was measured through PHM99 LAB pH meter. Total VFA concentration was determined by a gas-chromatograph (HP 5890 series II). The methane content in the CSTR reactor was measured with GC-TCD (MGC 82-12, Mikrolab a/s, Denmark).

Microbial analysis. Four time points with triplicate samples, taken under different TAN levels (before and after ammonia acclimation), were chosen for identifying the microbial dynamics

(Fig.1a). After an extra cleaning step with Phe:Chl:IAA (Sigma-Aldrich), genomic DNA was extracted from the samples according to PowerSoil® DNA Isolation Kit (MO BIO laboratories Inc., Carlsbad, CA USA). PCR amplification using primers 515F/806R was conducted on the V4 regions of 16S rRNA gene, and further sequencing was performed by Illumina MiSeq platform (Ramaciotti Centre for Genomics, Kensington, Australia). The raw sequences were analysed using CLC Workbench software (V.8.0.2) with Microbial genomics module plug in. Operational taxonomic units' (OTUs) phylogenetic assignment and alignment were well descripted in a previous study (Kougias et al., 2017). Alpha diversity was measured based on the Chao 1 bias-corrected index, while beta diversity was represented as Principal Component Analysis (PCA) using STAMP software.

Calculations and statistics

Free ammonia was calculated according to the equilibrium equation provided in a previous study (Angelidaki & Ahring, 1993). Statistical analyses and the plotted data were made using the OriginLab program. Student's t-test was used for estimation of statistically significant difference.

RESULTS AND DISCUSSION

Before the acclimation process started (days 0-28), the CSTR reactor was under steady state with an uninhibited methane yield of 332±16 NmL CH_4 g^{-1} VS (Fig.1a). Regardless of the TAN increase during the acclimation process (days 29-132), methane production remained statistically stable ($p>0.05$) throughout the whole experimental period, with an average methane production yield of 328±23 NmL CH_4 g^{-1} VS. The result clearly demonstrated that no ammonia inhibition was observed during the acclimation period, even at extremely high ammonia levels (10.5 g NH_4^+-N L^{-1}). A possible explanation is that, even though the TAN increased by 1.8-fold, the FAN kept stable (around 900 mg NH_3-N L^{-1}) because of the pH drop after acclimation started (Fig. 1b). During the acclimation period, the maximum VFA concentration was always below the suggested VFA inhibition threshold of 1500 mg/L during continuous AD process (Angelidaki et al., 2005) and pH (7.7-8.4) was also within the normal range for continuous biomethanation process (Lay et al., 1998). To our knowledge, this was the first time that a successful continuous AD process was achieved at such high ammonia levels using protein-rich microalgae as primary substrate.

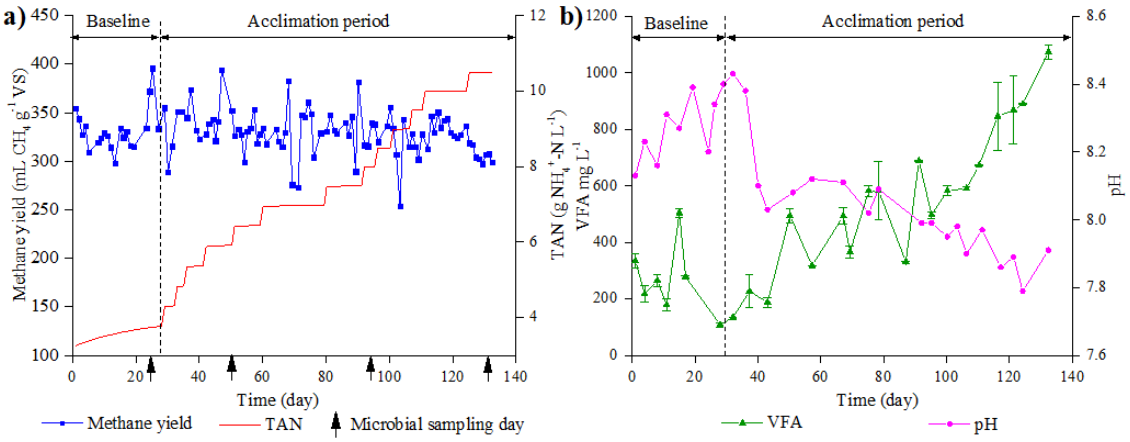

Figure 1. a) Methane yield and TAN change, b) VFA and pH variation throughout the experimental period, error bars denote standard deviation from the mean of triplicate measurements ($n=3$).

Alpha diversity (Fig.2a) and Beta diversity (Fig.2b) showed high dynamicity and subsequent changes in microbial communities during this acclimation process. The microbial diversity decreased significantly alongside with the increased TAN concentrations, indicating that only part of the initial microbes can survive under high ammonia toxicity levels and thus a more narrowed and specialized community was formed. Beta diversity also showed that with the higher ammonia

levels a bigger difference in communities was developed compared to the initial community before acclimation. This is due to the reduced microbial species and to the significant changes of relative abundance of the dominant species.

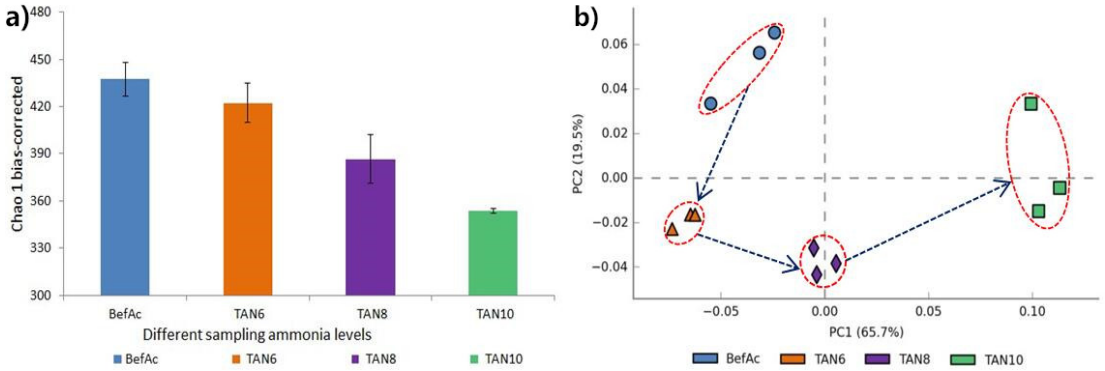

Figure 2. a) Alpha diversity and b) beta diversity at different experimental periods. BefAc, TAN6, TAN8 and TAN10 stands for before acclimation and TAN level at 6, 8 and 10 g NH_4^+-N L^{-1}, respectively.

CONCLUSIONS

The stepwise acclimation strategy used in present study allowed stable continuous biomethanation process at extremely high ammonia levels, which evidenced the possibility of protein-rich microalgae degradation through AD and also paved the way forward to the use of other protein rich substrates as anaerobic digestion feedstocks. Moreover, this study revealed that the microbial diversity decreases throughout the ammonia acclimation process.

ACKNOWLEDGMENTS

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